

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 September 2003 (04.09.2003)

PCT

(10) International Publication Number
WO 03/072613 A1

- (51) International Patent Classification⁷: **C08B 37/10**, A61L 33/08 (74) Agent: **BARCHIELLI, Giovanna**; Nicox Research Institute S.r.l, Via Ariosto, 21, 20091 Bresso- MI (IT).
- (21) International Application Number: PCT/EP03/02093 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 28 February 2003 (28.02.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 02290492.4 28 February 2002 (28.02.2002) EP
- (71) Applicant (*for all designated States except US*): **NICOX S.A.** [FR/FR]; 2455, routes des Dolines, F-06906 Sophia Antipolis Cedex (FR).
- (72) Inventors; and (75) Inventors/Applicants (*for US only*): **BOLLA, Manlio** [IT/IT]; Via Monte Popera, 11, I-20138 Milano (IT). **MONOPOLI, Angela** [IT/IT]; Via Cola di Rienzo, 4, I-20144 Milano (IT). **DEL SOLDATO, Piero** [IT/IT]; Via E. Toti, 22, I-20152 Monza (IT). **TORRI, Giangiacomo** [IT/IT]; Via G. Colombo, 81A, I-20133 Milano (IT). **NAGGI, Annamaria** [IT/IT]; Viale Cadorna, 27, I-20025 Legnano (IT). **CASU, Benito** [IT/IT]; Via G. Colombo, 81A, I-20133 Milano (IT).
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Declaration under Rule 4.17:**
— *of inventorship (Rule 4.17(iv)) for US only*
- Published:**
— *with international search report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 03/072613 A1

(54) Title: NITRO-DERIVATIVES OF EPOXYHEPARIN

(57) Abstract: The present invention relates to heparin, heparin fragments and heparin mimetics, functionalized by introduction of nitro groups and formation of epoxy groups. It also relates to the process for their preparation comprising the following steps: a. introduction of an epoxy functionality on positions 2,3 of the uronic acid; b. nitration of the epoxyheparin obtained in step a.

Nitro-derivatives of Epoxyheparin

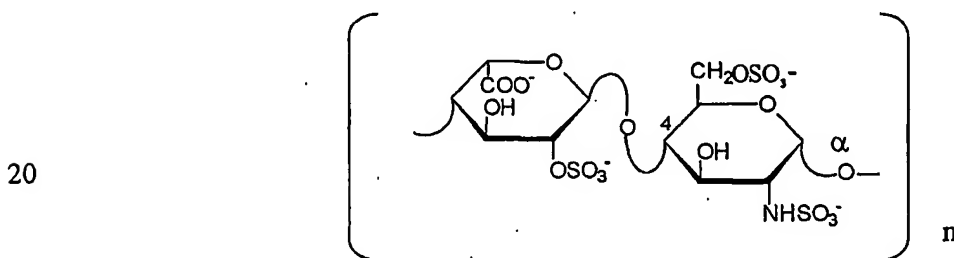
Technical Field

The present invention relates to new functionalized heparin for use in the preparation of materials presenting an anti-proliferating activity in surgical devices.

The compounds according to the invention are derivatives of heparin functionalized by formation of epoxide groups and nitro groups.

Background Art

It is known the anti-thrombotic activity of heparin, that makes it suitable to come in contact with biological fluids or tissues. Heparin is constituted by disaccharidic repetitive unities containing a residue of uronic acid and a residue of hexosamine. The prevalent repetitive unities of heparin are represented in scheme 1 wherein n usually varies from 8 to 35.



Schema 1: prevailing structure of heparin

25 It is known the use of heparin as agent to be supported on the surface of polymer materials in order to improve their compatibility with organic tissues or fluids. For example in US 4,987,181 depolymerized heparin is covalently bonded to an olefin copolymer. The resulting product is then used as adhesive film to be applied on polymeric surfaces that must come in contact with organic fluids or tissues. In
30 such process the depolymerization is useful to create the functional group necessary to the formation of the covalent bond, but it also gives rise to the

decrease of some biological activities, such as the anti coagulant activity. In US 5,541,305 it is obtained a material compatible with blood by heparinizing with alkali salts of heparin sulphate groups a polymer having quaternary amino groups. WO 99/27976 discloses a polymer material with high compatibility with organic fluids and tissues comprising a basic polymer which is covalently bonded to molecules of glycosaminoglycans. The glycosaminoglycans are bonded to the polymer through functionalization by formation of amino or epoxy groups.

The functionalization of polysaccharides with groups capable of releasing nitric oxide has been proposed in US 6,261,594. It discloses a nitric oxide donor comprising a modified chitosan polymer and a nitric oxide dimer (N_2O_2) directly bonded to the polymer backbone.

The object of the present invention is the provision of new functionalized heparin comprising epoxy functionalities and nitro functionalities. These compounds are easily fixed onto surfaces comprising functional groups and make them not only highly compatible with organic fluids or tissues, but also active against cell proliferation that takes place after surgical insertion of the same in the human body.

Another object of the present invention is the provision of a simple process for the production of said materials.


Disclosure of the Invention

The compounds object of the present invention having anti-inflammatory, non thrombogenic surfaces with an enhanced compatibility with organic fluids and tissues, are heparin, heparin fragments or heparin mimetics functionalized by introduction of nitro groups and formation of epoxy groups.

The epoxy group can be used to covalently bond heparin to a substrate which contains functional groups (e.g. hydroxyl groups).

The covalent bond is preferably formed through an amination reaction of the epoxy groups obtained on the uronic acid residues with ammonia or primary amines; more preferably the amination reaction is made with ammonia.

In alternative, the polysaccharide can be covalently bonded to the basic polymer through the use of spacers of general formula $T-R^{II}-T$ wherein: R^{II} is selected from the group comprising: linear or branched C_1-C_{20} alkylene, C_4-C_{20} cycloalkylene, C_6-C_{20} arylene, C_7-C_{20} arylalkylene; T is selected from the group

5 comprising: NR^I_2 , $Si(OR^I)_3$, COR^{III} , OR^I , X,  wherein R^{III} is selected from the group comprising: Cl, Br, I.

T can also represent a group from which through simple reactions it is possible to obtain one of the groups described, in case the compound $T-R^{II}-T$ does not prove to be stable. For example compounds of formula $NH_2-(CH_2)_nCHO$ wherein n
10 varies from 2 to 20 do not prove to be stable as they would tend to polymerize, it is possible anyway to use compounds of formula $NH_2-(CH_2)_nCH(OEt)_2$ wherein the aldehydic group is present as an acetal and can be restored through hydrolysis. Examples of groups $T-R^{II}-T$ are: diaminobutane, diaminopentane, diaminohexane, diaminoheptane, 1,4(aminoethyl)benzene, 1,4 diaminobenzene, 1-amino-4-
15 trimetoxysilylbutane, 1-amino-4-trimetoxysilylpentane, 4-methyl(trimetoxysilyl)-benzylamine, ethylenglycol, 1,6-dihydroxyhexane, 1,4-dibromomethylbenzene.

Preferred spacers are compounds of general formula $NH_2-(CH_2)_n-NH_2$ wherein n varies from 1 to 12, more preferably from 3 to 8.

The process for the preparation of compounds according to the invention
20 comprises the following steps:

- a. introduction of an epoxy functionality on positions 2,3 of the uronic acid;
- b. nitration of the epoxyheparin obtained in step a)

Though the temperature in step a) is not critical, it preferably varies between 10°C
25 and 80°C, more preferably between 40°C and 70°C; the reaction time preferably varies between 10 minutes and 3 days.

In the epoxidation reaction, the quantity of groups that reacted in comparison with the total quantity of reactive groups is indicated as a percentage. The used functionalization percentage influences whether and how heparin is bonded to the
30 basic polymer, but it also influences the biological activity of the obtained

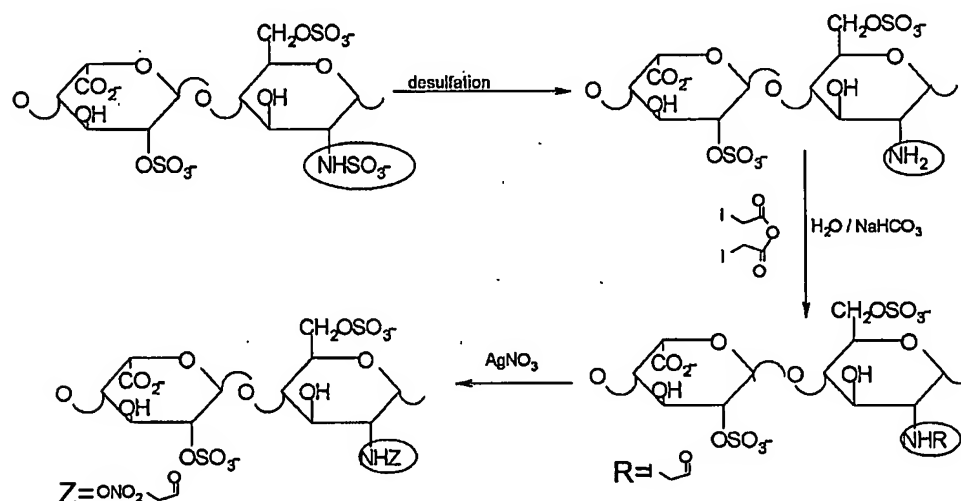
polymer material. The preferred percentage of groups that reacts in the epoxidation reactions is comprised between 5 and 60%, more preferably 10-40%. The nitro group can be covalently bonded to the saccharide structure either directly (e.g. through nitration of a carbon of the saccharide unit) or through a
5 divalent radical acting as a spacer between the saccharide unit and the nitro group. Suitable spacers are divalent radicals derived from C₂-C₂₀ aliphatic or aromatic hydrocarbons, ethers, polyethers, carboxylic acids or their derivatives, and the like.

A first path to the preparation of nitro-epoxyheparin according to the invention is
10 by direct nitration by means of a nitrating mixture. The reaction is preferably carried out by first preparing the nitrating mixture, wherein the previously dried epoxy-heparin is added in small amounts under stirring. Examples of nitrating mixtures are sulfuric acid-nitric acid, phosphoric acid-nitric acid, acetic anhydride-nitric acid, nitrous oxide-sulfuric acid. Preferred mixtures are the
15 mixture sulfuric acid-nitric acid, also called sulfo-nitric mixture and the mixture acetic anhydride-nitric acid. More preferred mixture is the mixture acetic anhydride-nitric acid.

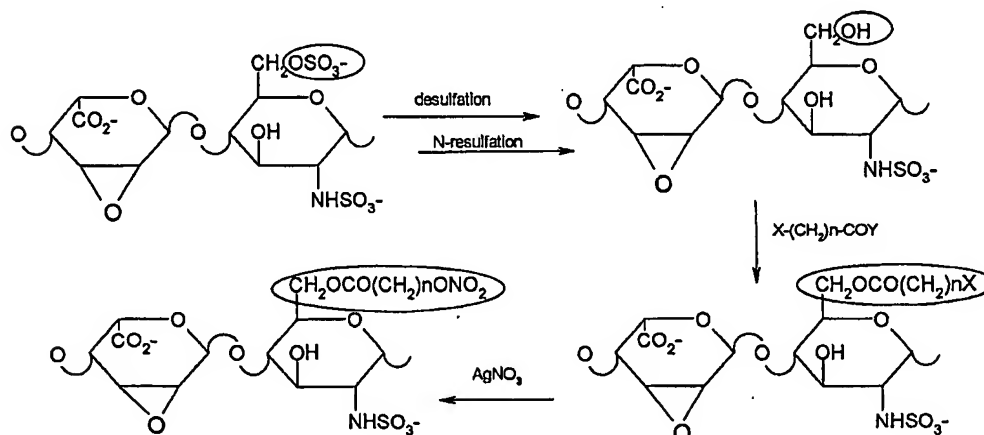
Using the latter mixture, the molar ratio between acetic anhydride and nitric acid can vary in a broad range and is preferably comprised between 1:1 and 1:2, more
20 preferably between 1:1.4 and 1:1.6. The amount of nitro groups per saccharide unit also varies considerably, and the ratio in equivalents between saccharide units and nitro groups is preferably comprised between 0.2 and 1.0, more preferably comprised between 0.3 and 0.8.

Another synthetic path for the synthesis of nitro-epoxyheparin according to the
25 invention is by haloacylation followed by nitration. In this case, it is possible to prepare first epoxy-heparin containing N-desulfated hexosamine. The N-desulfated hexosamine is then reacted with a halo anhydride such as iodoacetic anhydride, obtaining the corresponding amide in position 2 of the hexosamine. The iodine atom is then substituted with a nitro group by reaction with a suitable
30 nitrate. By using the suitable reaction conditions, it is possible to dose the amount of N-desulfation and, consequently, the amount of nitro groups introduced.

Examples of suitable nitrates are AgNO_3 and NBu_4NO_3 . It is possible to use anhydrides different from iodoacetic anhydride, such as the anhydride of a ω -halocarboxylic acid, obtaining in this way nitro-epoxyheparin having a different spacer group. Preferred halogens are iodine and bromine. More preferred is iodine. Using heparin fragments and iodoacetic anhydride as starting materials, the reaction scheme for a non epoxidized disaccharide unit is the following:

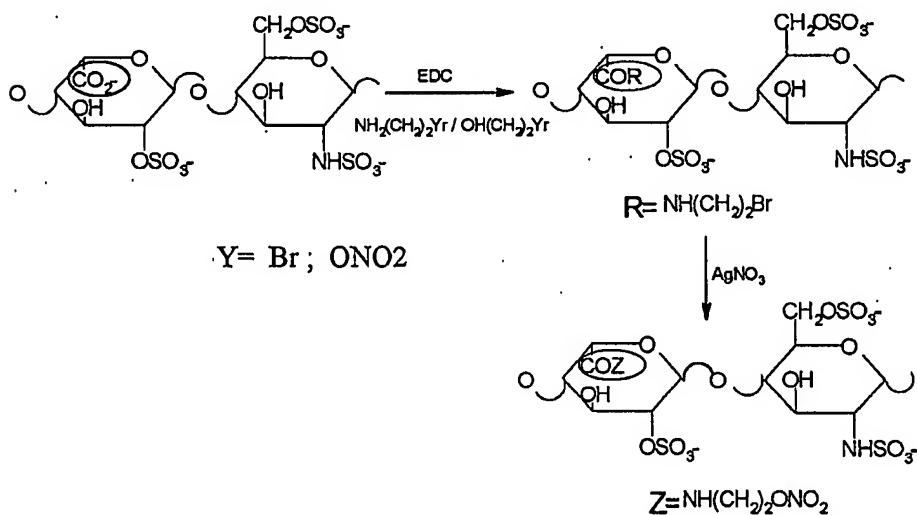


It is also possible to acylate the position 6 of desulfated hexosamine. In this case it is preferable to use compounds of formula $\text{X}-(\text{CH}_2)_n-\text{COY}$ wherein X is chlorine, bromine, iodine or tosyl; preferably X is bromine; Y is chlorine, mercaptothiazole, mercaptobenzothiazole, mercaptobenzoxazole, p-nitrophenol. The reaction scheme for an epoxidized disaccharide unit is the following:



Another possible route of preparation of nitroderivatives is described in the following scheme, where the carboxyl group is activated by reaction with di cyclohexylcarbodiimide and then reacted with a compound of general formula $X(CH_2)_nY$ where Y is Cl, Br, I, O-tosyl, ONO_2 and X is OH or NH_2 :

5



10

The nitro groups are measured by using the ratio functional group/saccharide, i.e. it is expressed in terms of nitro groups per saccharide ring. This ratio influences the biological activity of the obtained heparin. Depending on the final use of the compounds, it is possible to use high MW heparin ($10,000 < Mw < 18,000$), low MW heparin ($3,000 < Mw < 10,000$) or very low MW heparin ($Mw < 3,000$). The latter also includes heparin mimetics, i.e. synthetic oligosaccharides which reproduce sequences present in natural heparin (see for example EP 84999 and EP 300099).

The present invention is now more deeply described through examples that must be considered explanatory but that are not intended to limit in any way the invention itself.

20

Experimental section

The reactions have been carried out on the following substrates:

5 1. epoxyheparin 30%

5 g of heparin is dissolved in 63 ml of a solution NaOH 1 N. The solution is left under stirring stir for 45 min at 60°C, cooled and neutralized with diluted HCl. Then, the solution was dialyzed dialysis tubes with a 2000Da cut-off against water for 78 hours.

10 % epoxy group: 30% by ^{13}C NMR

2. epoxyheparin 24%

1 g of heparin is dissolved in 12 ml of a solution NaOH 1 N. The solution is left under stirring for 45 min at 60°C, cooled and neutralized with diluted HCl. Then, the solution was dialyzed dialysis tubes with a 2000Da cut-off against water for 78 hours.

15 % epoxy group: 24% ^{13}C NMR

Example 1

Preparation of nitro-epoxyheparin via nitration of epoxy-heparin 30% with nitro-acetic mixture

20 Nitroacetic mixture was prepared by dropping, over 45 minutes, 20.8 ml of cold acetic anhydride in 31.2 ml of nitric acid (90%) kept under stirring and cooled at -20°C in an N_2 /acetone bath. At the end, 130 mg of HEP were added. When the temperature was stabilized, N_2 /acetone bath was changed with an ice bath; the

25 mixture was stirred 4 hours at 0° C. The mixture was poured into 100 ml of a mixture diethyl ether – n-hexane 1:1 at -40°C in an N_2 /acetone bath. After 1 hour the sample is filtered, the precipitate dissolved in water, neutralized with a solution of diluted NaOH. The sample is dialyzed in dialysis tubes with a 2,000 Da cut-off against water for 78 hours. At the end, the sample is concentrated

30 through evaporation at a reduced pressure and at a low temperature. The product is purified by gel filtration on Sephadex G25.

The product has the following characteristics:

Ethero correlate NMR spectra presence of a signal at : 83 ppm; 5,5 ppm. The amount of epoxy groups is still 23%.

Elemental analysis: C: 22.51 %; N: 5.71 %;

5 N per saccharide: 1.3

ONO₂ per saccharide: 0.8

Yield = 72.5%

Example 2

10 Derivatization of nitro-epoxy-heparins with Diaminobutane.

To a 4% solution of nitro-epoxy-heparin of example 1 (50 mg in 1.25 ml of water) 12.5 µl of DAB is added and it is left for 50 hours at RT. The solution is precipitated with EtOH (4v/v), after adding 37 mg of NaCl in order to ease the precipitation. The sample is dialyzed in dialysis tubes with a 1000Da cut-off
15 against water for 48 hours. At the end, the sample is concentrated through evaporation at a reduced pressure and at a low temperature.

Claims

1. Polysaccharide characterized in that it is constituted of heparin, heparin
5 fragments and heparin mimetics, functionalized by introduction of nitro
groups and formation of epoxy groups.
2. Polysaccharides according to claim 1 wherein the percentage of
epoxidation is comprised between 5 and 60%.
3. Polysaccharide according to claims 1-2 wherein the ratio in equivalents
10 between saccharide units and nitro groups is comprised between 0.2 and
1.0.
4. Polysaccharides according to claims 1-3 wherein the polysaccharide is
heparin having Mw comprised between 10,000 and 18,000 Da.
5. Polysaccharides according to claims 1-3 wherein the polysaccharide is an
15 heparin fragment having Mw comprised between 3,000 and 10,000 Da.
6. Polysaccharides according to claims 1-3 wherein the polysaccharide is an
heparin fragment having Mw lower than 3,000 Da.
7. Polysaccharides according to claims 1-3 wherein the polysaccharide is an
heparin mimetic
- 20 8. Process for the preparation of polysaccharides according to claim 1
comprising the following steps:
 - a. introduction of an epoxy functionality on positions 2,3 of the
uronic acid;
 - b. nitration of the epoxyheparin obtained in step a.
- 25 9. Process according to claim 4 wherein step b. is performed using a mixture
acetic anhydride-nitric acid.
10. Process according to claim 5, wherein the molar ratio between acetic
anhydride and nitric acid is comprised between 1:1 and 1:2.

INTERNATIONAL SEARCH REPORT

Intern: .pplication No
PCT/EP J3/02093

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08B37/10 A61L33/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C08B A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 165 919 A (MASATOMI SASAKI ET AL.) 24 November 1992 (1992-11-24) *the whole document*	1-10
Y	US 5 691 423 A (DANIEL J. SMITH ET AL.) 25 November 1997 (1997-11-25) *the whole document*	1-10
Y	WO 99 27976 A (BAXTER BIOTECH TECHNOLOGY) 10 June 1999 (1999-06-10) cited in the application example 7	1-10

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the International search

3 June 2003

Date of mailing of the International search report

13/06/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx: 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Lensen, H

INTERNATIONAL SEARCH REPORT

Intern: plication No
PCT/EP 03/02093

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5165919	A	24-11-1992	JP 1244763 A	29-09-1989
			JP 2561309 B2	04-12-1996
			AU 621873 B2	26-03-1992
			AU 3347389 A	16-10-1989
			DE 68922726 D1	22-06-1995
			DE 68922726 T2	04-01-1996
			EP 0407580 A1	16-01-1991
			WO 8909069 A1	05-10-1989
US 5691423	A	25-11-1997	US 5405919 A	11-04-1995
			AU 695579 B2	13-08-1998
			AU 5448396 A	30-10-1996
			CA 2216696 A1	17-10-1996
			EP 0822833 A1	11-02-1998
			JP 11503456 T	26-03-1999
			WO 9632136 A1	17-10-1996
			US 6110453 A	29-08-2000
			US 2002119115 A1	29-08-2002
			US 5525357 A	11-06-1996
			US 5650447 A	22-07-1997
			US 6290981 B1	18-09-2001
			US 5632981 A	27-05-1997
			US 5718892 A	17-02-1998
			US 5676963 A	14-10-1997
			US 5910316 A	08-06-1999
			US 6379660 B1	30-04-2002
WO 9927976	A	10-06-1999	IT MI972644 A1	28-05-1999
			AU 1489199 A	16-06-1999
			WO 9927976 A1	10-06-1999